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EFFECT OF PROTEIN CONCENTRATION ON THE BINDING OF DRUGS TO HUMAN SERUM ALBUMIN—I. SULFADIAZINE, SALICYLATE AND PHENYLBUTAZONE

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Abstract—The binding of sulfadiazine, salicylate or phenylbutazone to human plasma and human serum albumin has been studied as a function of protein concentration. Results show that, as the concentration of albumin increases, the binding of all three drugs decreases. This phenomenon does not appear to be due to the presence of endogenous-competing ligands in serum or crystalline albumin preparations. Fluorescence depolarization measurements of dansylglycine complexes with human serum albumin show no evidence for the "molecular aggregation" of human serum albumin at high concentrations. Binding parameters obtained with dilute albumin solutions when extrapolated to physiological albumin concentrations may predict a higher degree of binding than that which is observed by direct measurement. The pharmacokinetic implications of these findings are discussed.

Klotz and Urquhart [1] were the first to report that ligand binding to plasma albumin is dependent on protein concentration. Their experiments with the methyl orange–bovine serum albumin (BSA) system clearly showed a very substantial decrease in complex formation when the albumin concentration was increased from 0.2 to 1 per cent. Klotz and Urquhart postulated that the activity coefficient of the species PA_{i-1}, where P represents the protein and A the methyl orange anion, must be decreased more than those of PA_i when the albumin concentration is increased. More recently, Brunkhorst and Hess [2] have reported a marked decrease in cortisol binding to human serum albumin (HSA) and BSA as the albumin concentration was increased from 0.4 to 4 per cent. These workers suggested that the presence of endogenous inhibitors might cause a reduction in ligand binding at the higher albumin concentrations. Shen and Gibaldi [3] have observed a decrease in thiopental binding to BSA at high protein concentrations and have attributed this phenomenon to a concentration-dependent "molecular aggregation" of albumin. Progesterone [4], the prostaglandins [5], tryptophan [6], propranolol [7] and sulfonamidochlorobenzoic acid [8] are other drugs which exhibit reduced binding to serum albumin when the concentration of the protein is increased.

In this paper, the effects of albumin concentration on the binding of salicylate, sulfadiazine and phenylbutazone have been studied. The mechanism of the observed decrease in binding of these drugs at high albumin concentrations has been examined by means of fluorescence polarization.

MATERIALS AND METHODS

Samples of whole blood were withdrawn by venipuncture, with informed consent, from normal, healthy subjects. The samples were immediately centrifuged, and the plasma was pooled and stored frozen at −20° until use. The thawed plasma was diluted with phosphate buffer, 0.1 M, pH 7.4, to give solutions ranging in concentration from approximately 4 to 0.04 per cent albumin.

Crystalline human serum albumin (Miles Laboratories, Elkhart, IN, Batch No. 33) was dissolved in sodium phosphate buffer, 0.1 M, pH 7.4, to give solutions ranging in concentration from 4 to 0.04 per cent. Reaction of HSA with dansyl chloride was carried out by the method of Chen [9]. The charcoal procedure of Chen [10] was used to remove fatty acids.

Binding studies. Aliquots of plasma or HSA solution [1–5 ml] were incubated for 30 min at room temperature (22°) with varying concentrations (100–500 μg/ml) of salicylate, sulfadiazine or phenylbutazone. A sample of this solution was removed for estimation of total drug concentration while the remainder of the sample was tested for bound drug by ultrafiltration or dialysis.

The Amicon Multi-Micro Concentrator (MMC) unit equipped with PM10 membranes (10,000 MW cutoff) was used in the ultrafiltration studies. Samples of protein–drug solution were placed in the cells, using a Pasteur pipette or disposable syringe. Each cell was individually operated and stirred. Ultrafiltration was carried out using nitrogen gas pressure of approximately 30 psi and 0.1 to 0.3 ml of ultrafiltrate was collected. Aliquots of ultrafiltrate and original protein–drug solution were analyzed for free and total drug concentration. Salicylate was estimated by the method of Trinder [11], sufadiazine by the method of Bratton and Marshall [12] and phenylbutazone by the method of Andreason [13].

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For equilibrium dialysis, a multicompartment dialysis system (Chemical Rubber Co., Cleveland, OH) consisting of two plastic blocks, each with five wells, was employed [14]. Washed dialysis tubing was cut into a single thickness and placed between the plastic blocks, which were then clamped together with bolts and screws. Samples of protein–drug solution were added to one side of the membrane, and sodium phosphate buffer, 0.1 M, pH 7.4, was added to the other. The sampling ports were then sealed, and the unit was gently agitated overnight at room temperature. It was determined that this procedure allowed complete equilibration. The rate of approach to equilibrium was found to be independent of albumin concentration [15].

Binding data were analyzed by the method of Scatchard [16] using the relationship.

$$(r/D) = Kn - Kr \qquad (1)$$

where r = number of moles of drug bound/mole of albumin, D = molar concentration of free drug and n = number of drug-binding sites/mole of albumin. When the plots were curved, it was assumed that there were multiple noninteracting sites. The values of n and K were obtained with the aid of the MLAB program operating on a PDP-10 computer [17]. The same program was also used to generate the figures which were drawn with a Calcomp plotter.

Fluorescence depolarization studies. The fluorescence depolarization of HSA solutions (0.4 and 4%) containing dansylglycine (10^{-5} and 10^{-6} M) and of solutions of dansyl–HSA conjugates was measured over a temperature range of 5–45° in an Aminco–Bowman spectrophotofluorometer modified according to the design of Chen and Bowman [18]. The cell compartment temperature was controlled by a thermostatic water bath while the sample temperature was measured with an Omega Engineering Inc. telethermometer.

Apparent relaxation times, ρ_h, were calculated from the Perrin–Weber equation [19]

$$1/P - 1/3 = (1/P_0 - 1/3)\,(1 + 3\tau/\rho_h) \qquad (2)$$

where P = observed polarization, and P_0 = limiting value of polarization in the absence of Brownian motion. The value of P_0 was calculated by extrapolation of a plot of $1/P$ vs T/η (where T = absolute temperature and η = coefficient of viscosity) to infinite viscosity (i.e. $T/\eta = 0$) and τ = fluorescence lifetime.

Unless otherwise stated, all solutions contained 0.1 M sodium phosphate buffer, pH 7.5.

RESULTS

The Scatchard plots for binding, of phenylbutazone, salicylate and sulfadiazine at a fixed concentration of 250 μg/ml, to various dilutions of plasma are shown in Fig. 1. It can be seen clearly that in each case the plot has a positive slope which is in contrast to the negative slope predicted by equation 1. Similar plots were obtained by Brunkhorst and Hess [2] for the cortisol–albumin system and by Shen and Gibaldi [3] when they measured the binding of thiopental to HSA.

In order to examine this phenomenon more closely, the binding of all three drugs to purified HSA was measured at low (0.1%) and high (1.0%) protein con-

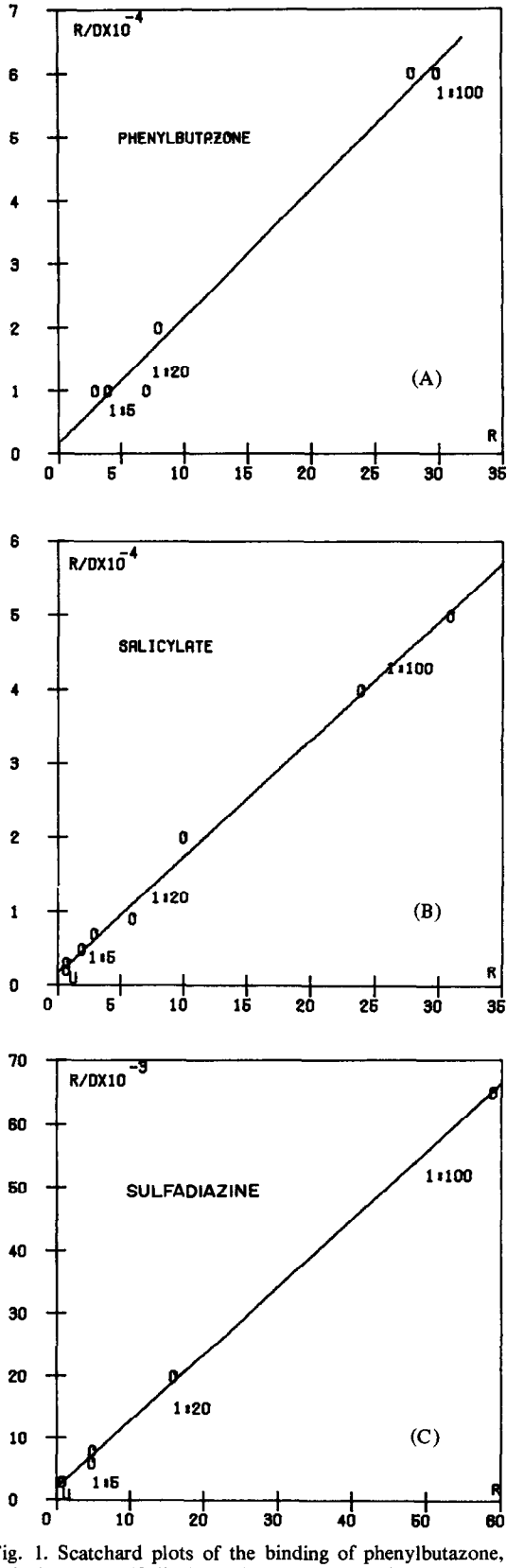
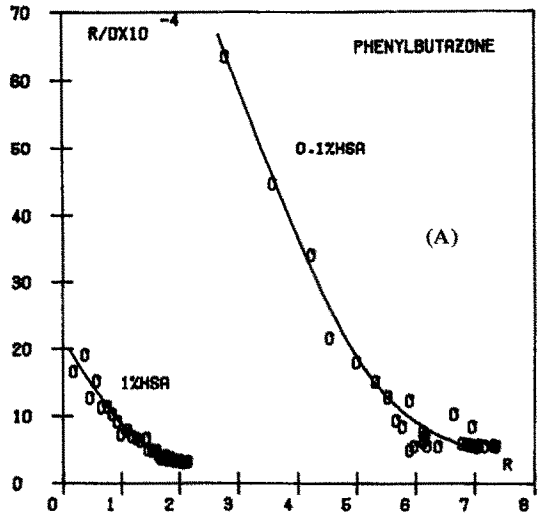
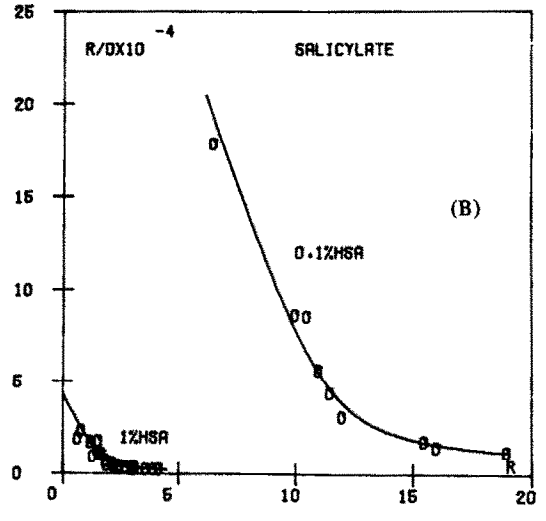


Fig. 1. Scatchard plots of the binding of phenylbutazone, salicylate and sulfadiazine to human plasma. The concentration of drug was held constant at 250 μg/ml while the plasma was either undiluted (U) or diluted (1:5, 1:20, 1:100) with 0.1 M sodium phosphate buffer (pH 7.4)





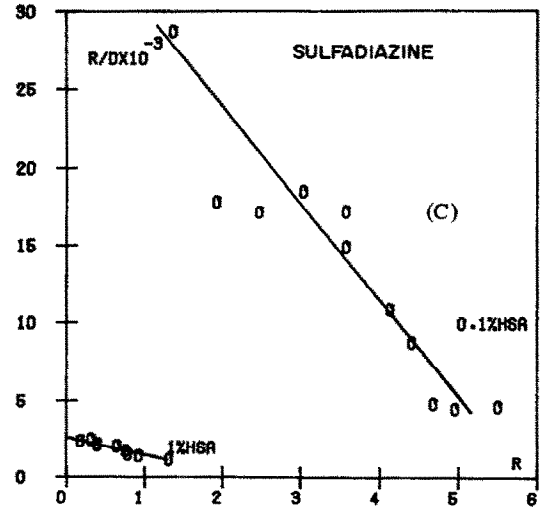
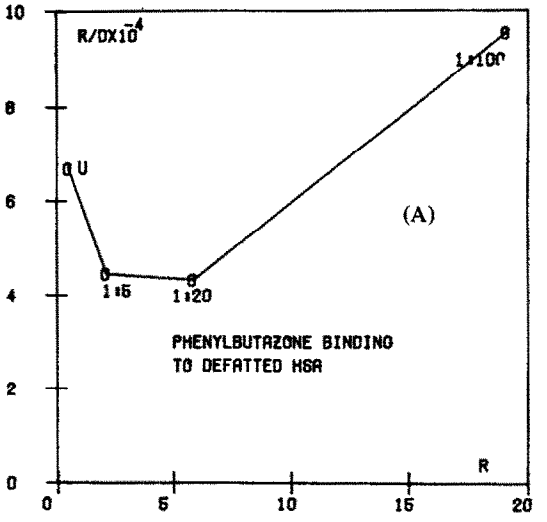
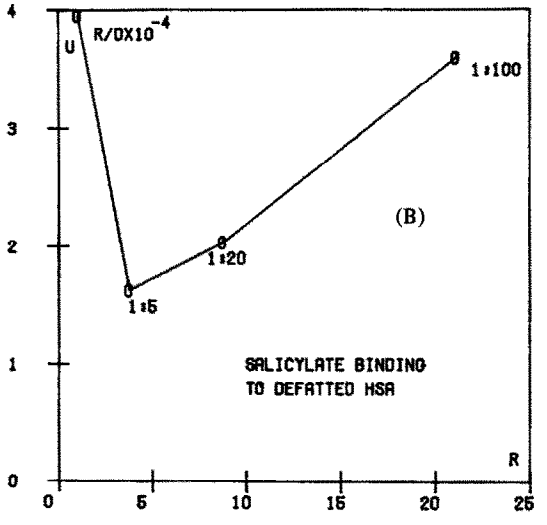


Fig. 2. Scatchard plots of the binding of phenylbutazone, salicylate and sulfadiazine to human serum albumin. The concentration of HSA was either 0.1 or 1% as indicated. All solutions contained 0.1 M sodium phosphate buffer (pH 7.4)





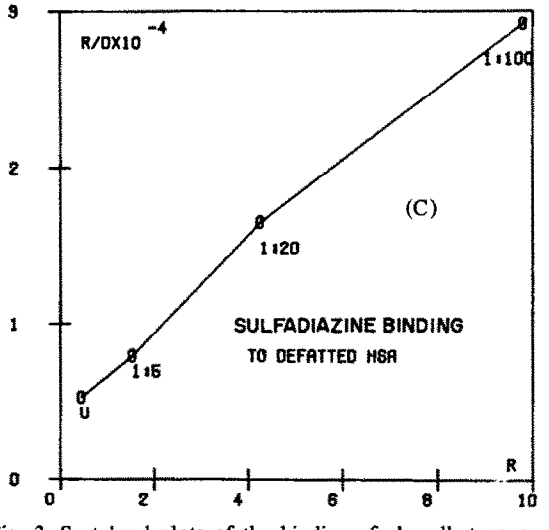


Fig. 3. Scatchard plots of the binding of phenylbutazone, salicylate and sulfadiazine to charcoal-treated human serum albumin. The concentration of drug was held constant at 250 μg/ml. The concentration of HSA in the undiluted sample (U) was 4%. All solutions contained 0.1 M sodium phosphate buffer (pH 7.4)

centrations. In these experiments, the protein concentration was held constant while the concentration of drug was varied. In contrast to the previous findings (Fig. 1). each Scatchard plot had the negative slope predicted by equation 1 (Fig. 2). However, estimation of the binding parameters from the respective plots (Table 1) indicated that, for phenylbutazone and salicylate, the number of binding sites increased dramatically with protein dilution while the affinities of the sites were not greatly changed. With sulfadiazine, which has a single class of binding sites, dilution of the HSA solution caused an increase in both n and K (Table 1).

One possible explanation for the anomalous binding observed in plasma could be the presence of constituents in plasma which can bind to albumin and reduce drug binding by a competitive mechanism [2]. Dilution of plasma to obtain the binding curves shown in Fig. 1 would result in dilution of such competitive agents, which in turn could lead to the observed increase in drug binding. However, when plasma was diluted with the ultrafiltrate from another plasma sample, the same increase in affinity was observed.

Chen [10] has found that commercial preparations of purified albumin contain significant amounts of bound fatty acids. Since fatty acids are known to compete with acidic drugs for binding sites on albumin [20], it is conceivable that the presence of bound fatty acids could account for the decreased binding of salicylate, phenylbutazone and sulfadiazine at high HSA concentrations (Fig. 2). Samples of HSA were therefore defatted by the method of Chen [10] and drug binding was measured as before. With HSA solutions at normal plasma concentrations (4%), removal of fatty acids appeared to increase the binding capacity of the albumin for both salicylate and phenylbutazone (Fig. 3). However, dilution of the albumin solutions beyond 1:5 again gave rise to a binding plot with positive slope. While the affinity of sulfadiazine for defatted HSA was increased, this drug did not show the biphasic effect seen with phenylbutazone and salicylate (Fig. 3).

The possibility that HSA might form "molecular aggregates" at high albumin concentrations was studied by means of fluorescence depolarization. Weber [19] has shown that changes in the conformation of BSA, induced by either urea or changes in pH, can be detected by measuring the fluorescence depolarization of dansyl–BSA conjugates. Previous studies have established that dansylglycine binds to HSA at the same site as many acidic drugs and that in doing so the fluorescence quantum yield of this ligand increases [18]. Polarization measurements were therefore made on 0.4 and 4% solutions of HSA containing dansylglycine.

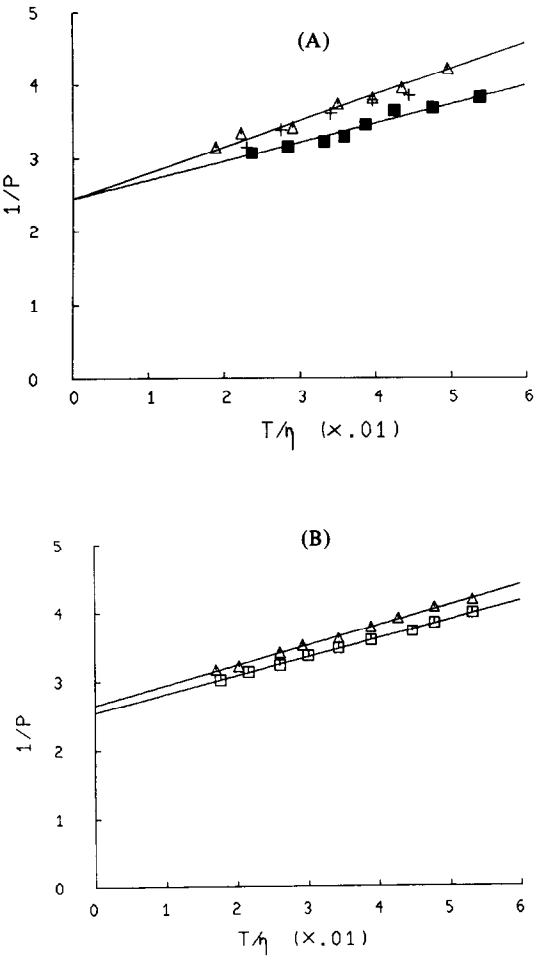


Fig. 4. Fluorescence depolarization. (A) Fluorescence depolarization of complexes between dansylglycine and human serum albumin. Key: —△——△—, 0.4% HSA + 10^{-6} M dansylglycine; + + , 0.4% HSA + 10^{-6} M dansylglycine + 6% sucrose; and —■——■—, 4% HSA + 10^{-5} M dansylglycine. Excitation and emission wavelengths were 380 and 450 nm respectively. T is the absolute temperature, η is the coefficient of viscosity (cP) and P is the observed polarization. All solutions contained 0.1 M sodium phosphate buffer (pH 7.5). (B) Fluorescence depolarization of dansyl-labeled human serum albumin. Key: —△——△—, 0.4% HSA; and —□——□—, 4% HSA. The 0.4% solution of HSA contained 1 dye/mole of protein. The 4% solution was prepared by adding unlabeled HSA to give the correct final protein concentration. Excitation and emission wavelengths were 380 and 450 nm respectively. All solutions contained 0.1 M sodium phosphate buffer (pH 7.4).

Table 1. Effect of human serum albumin concentration on the binding of salicylate, sulfadiazine and phenylbutazone *

Binding parameter	Salicylate		Sulfadiazine		Phenylbutazone	
	1% HSA	0.1% HSA	1% HSA	0.1% HSA	1% HSA	0.1% HSA
n_1	1.7	11.5	2.4	5.9	1.4	5.9
n_2	15.0	161.7			4.1	3.3
K_1 (M^{-1})	2.5×10^4	3.3×10^4	1.1×10^3	6.2×10^3	1.3×10^5	1.3×10^5
K_2 (M^{-1})	0.1×10^3	0.2×10^2			0.4×10^4	0.5×10^4

* Calculated from the Scatchard plots in Fig. 2.

Table 2. Fluorescence lifetimes of dansylglycine bound to HSA and a dansyl–HSA conjugate

Sample	Time
0.4% HSA + 10^{-6} M dansylglycine	17.7 nsec
0.4% HSA + 2.4×10^{-6} M dansylglycine	17.8 nsec
4% HSA + 10^{-5} M dansylglycine	16.6 nsec
4% HSA + 10^{-6} M dansylglycine	16.9 nsec
0.4% Dansyl–HSA conjugate*	16.2 sec
4% Dansyl–HSA conjugate	17.1 sec

* Contains two dansyl groups/mole of HSA.

The results, shown in Fig. 4A, indicate that the bound dansylglycine had a slightly higher polarization in the presence of the 4 per cent HSA solution. This difference did not appear to be the result of increased viscosity, since the addition of 6 per cent sucrose to the 0.4 per cent HSA solution (which would raise the viscosity of the solution to that of 4 per cent HSA) did not alter significantly the fluorescence depolarization of bound dansylglycine. The fluorescence lifetime of dansylglycine in 0.4 and 4 per cent HSA dilutions was the same (Table 2), suggesting that the observed differences in depolarization could not be attributed to different fluorescence lifetimes. The concentration of dansylglycine was also not a factor, since similar depolarization data were obtained with 10^{-6} and 10^{-5} M dansylglycine in the presence of 0.4 per cent HSA. Dansyl conjugates of HSA exhibited the same concentration-dependent depolarization differences as were observed with the dansylglycine complexes (Fig. 4B).

The limiting polarization value (P_0) for the dansylglycine–HSA system can be estimated from Fig. 4A to be 0.407. From this value and the measured fluorescence lifetimes in Table 2, it can be calculated from equation 2 that the apparent relaxation times, ρ_n, at 19° for dansylglycine in 0.4 and 4 per cent HSA are 115.4 and 145.5 nsec respectively.

DISCUSSION

The data presented here clearly show that the binding of phenylbutazone, salicylate and sulfadiazine to HSA is dependent upon protein concentration. It is obvious from a comparison of Figs. 1 and 2 that the anomalous Scatchard plots obtained with diluted plasma are due to the existence of a series of binding curves, each characteristic of a particular albumin concentration.

The explanation provided by Klotz and Urquhart [1] for the dependence of methyl orange binding on the concentration of BSA is a restatement of the problem in thermodynamic terms which does not provide any clue concerning the mechanism. The endogenous competitive inhibitor theory of Brunkhorst and Hess [2] and the molecular aggregation postulate of Shen and Gibaldi [3] represent attempts to provide a molecular basis for the experimental observations. The competitive inhibitor theory is an attractive one, since dilution of plasma in the presence of a constant concentration of drug would result in an increase in the drug/competitor ratio. At the same time, plasma dilution would cause dissociation of the inhibitor–HSA complex. Both of these phenomena would lead to increased drug binding to HSA as the protein concentration was lowered. However, dilution of plasma with plasma ultrafiltrate did not prevent the increase in drug binding at low protein concentrations. In addition, the dilution effect was also observed with crystalline HSA (Fig. 2). While HSA may be free of most of the endogenous ligands found in plasma, it is known that commercial preparations contain variable amounts of bound fatty acids, which may be removed by charcoal treatment at low pH [10]. Although fatty acid removal did increase the binding of phenylbutazone and salicylate in 4% HSA solutions (Fig. 3), dilution beyond 1:5 produced the same general increase in affinity. The binding of salicylate to HSA did not seem to be greatly affected by defatting (Fig. 3). Thus, it seems unlikely that inhibitory impurities play a significant role in the dependence of drug binding on albumin concentration.

Ray *et al.* [21] have reported that the binding of dodecylsulfate and dodecanol to BSA decreases as the protein concentration increases. However, subsequent experiments by Cassel *et al.* [15] have shown that these results were due to an anomalously slow approach to equilibrium by these ligands when binding was measured at high BSA concentrations by means of the equilibrium dialysis technique. In our equilibrium dialysis measurements no dependence of the rate of approach to equilibrium on HSA concentration was observed. Furthermore, Cassel *et al.* [15] also found no effect of BSA concentration on the rate of approach to equilibrium by the symmetrical picrate anion. Thus, it seems unlikely that our results are due to an experimental artifact.

If HSA undergoes some form of molecular aggregation at high protein concentrations, then it is conceivable that drug-binding sites could be occluded and that the affinity of exposed sites could change. It has been reported that, under certain circumstances, HSA will

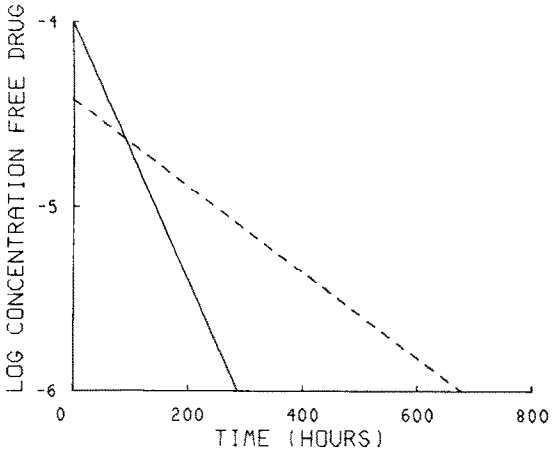


Fig. 5. Effect of albumin binding on the plasma concentration of free sulfadiazine. The computer-generated curves were derived from a pseudo one-compartment model [25, 26] using the binding parameters in Table 1. Key: ————, 1% HSA; and — — —, 0.1% HSA. The following assumptions were made: total plasma volume, 3 liters; total body water, 40 liters; concentration of HSA, 1×10^{-3} M; kidney clearance, 0.82 liter/hr; and initial dose of sulfadiazine 5 m-moles (1.25 g).

form dimers and higher polymers [22–25]. Indeed, commercial albumin is known to contain a significant amount of polymerized material, mainly in the form of dimers [25]. The fluorescence depolarization studies clearly show that, for example, at 19° the apparent ρ_h values for dansylglycine in 0.4 and 4 per cent HSA are 115.4 nsec and 145.5 nsec respectively. If it is assumed that HSA is a spherical molecule and that the dansylglycine is rigidly attached, then the rotational correlation time (T_c) [26] of the protein is 38.5 nsec in the 0.4 per cent solution but 48.5 nsec in the 4% solution. From the Stokes–Einstein relationship

$$T_c = (4\pi\eta a^3/3kT) \quad (3)$$

where k = Boltzmann constant, it can be shown that the difference in T_c values at the two protein concentrations can be attributed to an 8 per cent increase in the radius (a) of HSA. Thus, it seems highly unlikely that HSA in solution undergoes molecular aggregation as its concentration increases.

From a pharmacological standpoint, these observations are of considerable importance. Many workers who study drug binding use very dilute albumin solutions for their measurements. This is particularly true when sensitive spectroscopic techniques, e.g. fluorescence, are employed to measure binding. It is clearly incorrect to apply binding data obtained with dilute albumin solutions to the *in vivo* situation, where much higher protein concentrations are encountered. As an example, the binding parameters for sulfadiazine in Table 1 have been used to assess the effect of HSA binding on the elimination of the drug from the body according to the pseudo one-compartment model described by Krüger-Thiemer *et al.* [27]. In this model, it is assumed that (a) a single dose of drug equilibrates instantaneously between plasma and tissue water, (b) drug binding is only to plasma albumin and (c) elimination is solely via the kidney [28]. It can be seen from Fig. 5 that the data obtained with 1 per cent HSA predict a higher initial free concentration of sulfadiazine in the plasma (10^{-4} M vs 3.8×10^{-5} M) and a more rapid decline in plasma levels of free drug.

These studies have shown clearly that the binding of sulfadiazine, salicylate and phenylbutazone to HSA is inversely related to protein concentration. This phenomenon is not due to the presence of competing ligands and cannot be attributed to the molecular aggregation of HSA at high protein concentrations. The increase in binding at low HSA concentrations may explain the surprisingly high drug binding observed occasionally in patients with severe hypoalbuminemia [29].

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